The Formin FMNL3 Controls Early Apical Specification in Endothelial Cells by Regulating the Polarized Trafficking of Podocalyxin

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Summary
Angiogenesis is the fundamental process by which new blood vessels form from pre-existing vasculature. It plays a critical role in the formation of the vasculature during development, and is triggered in response to tissue hypoxia in adult organisms. This process requires complex and coordinated regulation of the endothelial cell cytoskeleton to control cell shape and polarity. In our previous work, we showed that the cytoskeletal regulator FMNL3/FRL2 controls the alignment of stabilized microtubules during polarized endothelial cell elongation, and that depletion of FMNL3 retards elongation of the intersegmental vessels in zebrafish [1]. Recent work has shown that FMNL3 is also needed for vascular lumen formation [2], a critical element of the formation of functional vessels. Here we show that FMNL3 interacts with Cdc42 and RhoJ; two Rho family GTPases known to be required for lumen formation. FMNL3 and RhoJ are concentrated at the early apical surface, or AMIS, and regulate the formation of radiating actin cables from this site. In diverse biological systems, formins mediate polarized trafficking through the generation of similar actin filaments tracks. We show that FMNL3 and RhoJ are required for polarized trafficking of podocalyxin to the early apical surface – an important event in vascular lumenogenesis.

Results and discussion

FMNL3 signals downstream of Cdc42 and RhoJ

Like other diaphanous-related formins (DRFs), FMNL3 contains a conserved Rho GTPase binding domain (GBD) that mediates targeting and activation [3]. To identify upstream regulator(s) of FMNL3 in angiogenesis, we used the yeast two-hybrid assay to screen the isolated GBD domain against the Rho GTPase family. Like the related formins FMNL1 and 2 [4, 5], FMNL3 interacted with activated Cdc42 (Figure 1A), but also RhoJ, RhoD, RhoH, and Rnd1. We focused on Cdc42 and RhoJ as both signaling proteins are required for angiogenesis [6-8]. We confirmed the interactions with FMNL3 by co-immunoprecipitation in HEK 293 cells. Full-length FMNL3 bound to Cdc42 and RhoJ in this assay, but not to RhoA, Rac1 or RhoC (Figures 1B, S1A). We next sought to link FMNL3 to known endothelial functions of Cdc42 and RhoJ. RhoJ controls focal adhesion disassembly in endothelial cells through regulation of the GIT-PIX complex [7, 9]. We silenced FMNL3 in primary endothelial cells (ECs; Figure S1B); however, this had no effect on focal adhesion number (data not shown), suggesting that FMNL3 is not involved in this aspect of RhoJ signaling. Cdc42 controls polarization of the Golgi in migrating ECs and in response to fluid flow [10, 11]. Silencing of FMNL3 significantly disrupted Golgi reorientation in a scratch polarization assay, although to a lesser extent than silencing of Cdc42 (Figure S1C). Depletion of FMNL3 had no significant effect on EC velocity or chemotaxis to VEGF, although it gave a slight increase in persistent movement (Figure S1D). We conclude that, while FMNL3 depletion affects polarization in ECs, it does not make a significant contribution to their directed migration towards VEGF.

FMNL3 controls actin cable formation at the early apical surface

Angiogenesis requires complex regulation of cell polarity. ECs first undergo polarized elongation in the direction of the pro-angiogenic signal to become tip cells, which then guide the migration of the following stalk cells [12]. ECs depleted of FMNL3 show inhibition of this polarized elongation [1, 13]. As the vessel matures, adjacent stalk cells undergo apical-

basolateral polarization perpendicular to this axis to begin the process of lumen formation [14, 15]. Recent work by Gerhardt and co-workers has shown that FMNL3 is required for vascular lumen formation in zebrafish [2]. We hypothesized that FMNL3 might be involved in polarity regulation during lumenogenesis and so investigated its role in formation of the apical-basolateral axis.

In epithelial cells, specification of the apical surface begins with formation of the apical membrane insertion site (AMIS), which is the target of polarized trafficking of early apical membrane proteins. Vesicles containing these components are delivered to a juxtamembrane location beneath the AMIS via transcytosis from the basolateral surface. Formation of the AMIS concentrates apical components prior to formation of tight-junctions. Once these are in place, the contents of the AMIS are released to the early apical surface to form the pre-apical patch. [16-18]. In spreading ECs undergoing apical-basolateral polarization, the AMIS is focused into an F-actin-rich apical bud [19]. FMNL3 was strongly localized to the AMIS (Figure 1C), but was dispensable for its formation; in fact, depletion of FMNL3 led to an increase in cells with an AMIS (Figure 1D). Live-imaging AMIS formation showed that cells depleted of FMNL3 initiated AMIS formation at the same time as control cells; however, the structure took significantly longer to disassemble (Figure 1E; Movie S1). Formins nucleate linear actin cables, and we observed linear actin cables radiating from the majority of endothelial AMIS (Figure 1F). Depletion of FMNL3 significantly reduced the number of cells with cables (Figure 1F), and significantly reduced cable number in cells where they did form (Figure 1G). Conversely, overexpression of FMNL3 shifted the distribution towards more cables (Figure 1G). We conclude that FMNL3 nucleates the formation of radiating actin cables at the endothelial AMIS.

FMNL3 regulates actin-based trafficking of podocalyxin to the early apical surface

In budding yeast, formins at the bud site produce linear actin cables that mediate polarized trafficking towards the bud, promoting bud growth [20-22]. Silencing of FMNL3 had no effect on AMIS size; however, raising the cellular concentration of FMNL3 caused a dramatic increase (Figure 1H), suggesting an analogous role for this formin in polarized trafficking to the pre-apical surface. To examine this, we measured the recruitment of a number of key proteins in AMIS formation. Silencing of FMNL3 did not affect recruitment of atypical PKCξ or Par3 (Figure 2A), early components of the apical polarity complex that are translocated to the plasma membrane via Cdc42 [23]. Recruitment of the exocyst subunit Sec8 and the junctional proteins VE-cadherin and β-catenin were similarly unaffected. Silencing of FMNL3 significantly reduced recruitment of podocalyxin (Figure 2A), an important factor lumen formation [24]. As reported previously [19], we observed podocalyxin in cytoplasmic vesicles clustered directly beneath the AMIS (Figure 2C). Silencing of FMNL3 had no effect on total cellular podocalyxin (Figure 2B), but significantly reduced both the amount of podocalyxin delivered to the AMIS, and its specific enrichment there (Figure 2C).

In polarizing epithelial cells, podocalyxin is trafficked from the basolateral surface to the AMIS through a Rab11-dependent pathway [16]. In polarizing ECs, podocalyxin+ vesicles co-localized with the early endosomal marker Rab5 and with Rab25 (Figure S2A, B), but not with Rab7, 8, or 11 (data not shown). We presume that the Rab5+ vesicles represent newly-endocytosed podocalyxin. Rab25 (alternatively called Rab11c) is related to Rab11 and controls the constitutive apical recycling of several proteins in epithelial cells [25]. We

conclude that podocalyxin is trafficked through a Rab25+ recycling pathway to the AMIS in ECs.

Podocalyxin vesicles showed some alignment with microtubules in the EC periphery (Figure 2D). Consistent with this, nocodazole significantly inhibited trafficking of podocalyxin to the AMIS (Figure 2G). Proximal to the AMIS, alignment of podocalyxin vesicles with microtubules was not apparent (data not shown). Instead, a subset of these vesicles appeared to be aligned with the actin cables, although the resolution of confocal microscopy was not sufficient to be certain. Stimulated emission depletion (STED) super-resolution microscopy showed a subset of podocalyxin vesicles aligned with actin cables proximal to the AMIS (Figure 2E). Rab25 can couple vesicles to actin filaments via the myosin Vb motor protein [26]. Myosin Vb was concentrated at the AMIS (Figure 2F), and treatment of cells with a specific inhibitor of myosin V (MyoVin-1; [27]) significantly inhibited trafficking of podocalyxin to the AMIS (Figure 2G). Neither nocodazole nor MyoVin-1 affected FMNL3 recruitment to the AMIS (Figure S2C). We conclude that both microtubules and actin are required for intracellular trafficking of podocalyxin during polarization, but that terminal transport to the AMIS is actin-based and dependent on myosin V.

RhoJ regulates polarized podocalyxin trafficking to the AMIS

Cdc42 is a critical organizer of apical polarity in epithelial and endothelial cells [12, 28] and is required for vascular lumen formation. Less is known about RhoJ, although recent studies have shown that depletion of RhoJ inhibits vascular lumen formation *in vitro* [29]. We examined the localization of both proteins with respect to the AMIS. Cdc42 was concentrated in vesicles proximal to the AMIS (Figure 3A). Previous studies have seen Cdc42 localized to the microtubule organizing center (MTOC) with the formin mDia2 [30]; however, we did not see MTOC staining here (Figure S3A). RhoJ was observed in vesicles near the AMIS, but also showed a marked concentration at the AMIS itself (Figure 3A). Depletion of either RhoJ or Cdc42 significantly reduced the formation of radiating actin cables at the AMIS (Figure 3B). Intriguingly, their depletion also delayed AMIS disassembly (Figure S3B). Depletion of RhoJ strongly inhibited trafficking of podocalyxin to the AMIS, whereas depletion of Cdc42 had negligible effect (Figure 3B). We conclude that FMNL3 acts downstream of RhoJ to mediate polarized trafficking of podocalyxin to the endothelial AMIS.

FMNL3 is required for targeting of podocalyxin to the apical surface during endothelial lumen formation

Trafficking of podocalyxin to the early luminal surface is an important step in the formation of the vascular lumen [31]. Podocalyxin is a sialomucin that contributes a highly negative charge to the apical glycocalyx. Its insertion into the apical surface leads to electrostatic repulsion between the opposing luminal faces, leading to opening of the luminal space [32]. We examined trafficking of podocalyxin during lumen formation using a well-characterized *in vitro* 3D co-culture model of angiogenesis [33-35]. In this assay, primary ECs are mixed with primary dermal fibroblasts. The fibroblasts secrete and condition a collagen-rich extracellular matrix through which the ECs grow. The ECs form an anastomosing 3D network of tubules over a period of 5 days. At the end of the assay, the tubules have a patent lumen, although this is mainly closed in the absence of flow [33]. We stained for the endothelial surface protein PECAM, which allowed us to identify the luminal surface between adjacent ECs due to the increased signal from two opposed plasma membranes (Figure 4A).

Podocalyxin localized strongly to the early luminal surface, as reported [31]; however, this polarized localization was lost in endothelial tubules depleted of FMNL3 and the protein was seen instead in intracellular vesicles (Figure 4A, B). We conclude that FMNL3 is required for the polarized trafficking of podocalyxin to the early apical surface during vascular lumenogenesis.

Recent studies reveal a striking conservation of formin function in the organization of polarized traffic across species. In the budding yeast *Saccharomyces cerevisiae*, the formin Bnr1p nucleates actin cables that direct secretory traffic towards the growing bud [36]. Comparable cables are nucleated by the plant formin FH5 to target trafficking to the tip of elongating pollen tubes in *Aradadopsis* [37]. In addition to such long-range polarized traffic, formins also control short-range trafficking near the polarized surface. In mammalian epithelial cells, the formin INF2 signals downstream of Cdc42 to regulate actin-dependent apical transcytosis. INF2 is concentrated to the apical surface and is required for lumen formation in epithelial cysts [38]. In *Drosophila* epithelial tubes, the formin Dia is localized to the luminal surface and is required for the secretion of a subset of apical cargoes. This formin is involved in formation of the terminal web, a dense array of actin filaments at the epithelial apical surface [39]. In mammalian epithelial cells, the orthologue mDia1 plays a similar role, and is responsible for the final stages of trafficking close to the apical surface [40]. This is analogous to the role of the formin Bni1p in budding yeast, which mediates the short-range trafficking of cargo within the bud itself [36]. The role of FMNL3 in EC lumen formation fits the category of long-range transport.

In their recent study, Gerhardt and co-workers showed that FMNL3 maintains a pool of dynamic F-actin filaments at EC junctions that is required for junctional stability during lumen formation [2]. This places FMNL3 in a coordinating role in lumen formation. In our model (Figure 4C), actin cables nucleated by FMNL3 and RhoJ at the AMIS direct the trafficking of podocalyxin to the early apical surface. The AMIS is rich in phospho-ERM proteins ([19, 31]; Figure S4), which physically interact with podocalyxin [41]. We propose that this tethers podocalyxin at the early apical surface at a stage of lumenogenesis when junctions are not fully mature [31], and cannot efficiently restrict proteins to the apical surface. The function of the long actin cables would be to direct podocalyxin trafficking from the whole basolateral surface to the AMIS, translocating and concentrating the protein. Stabilization of the endothelial junctions, promoted by FMNL3, would then be followed by release of podocalyxin into the apical membrane, allowing maturation of the lumen. In this way, FMNL3 would coordinate the delivery and incorporation of podocalyxin into the luminal surface. Intriguingly, in cells depleted of FMNL3, the AMIS takes longer to disassemble (Figure 1E), supporting this coordinating role. We propose that a similar formin-mediated regulation is likely to exist during epithelial lumen formation. Whether this is coordinated by FMNL3 or related formins is unclear at present.

Supplemental Information

Detailed experimental procedures, supplemental figures and a movie are available with this article online at:

Acknowledgements

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Figure legends

Figure 1. FMNL3 controls actin cable formation at the EC pre-apical domain.
(A) Interaction of the FMNL3 GBD with Rho family GTPases. Yeast were transfected with constitutively-active Rho GTPases and the FMNL3 GBD. Dual expression was selected on DDO medium. Interactions were detected by growth on TDO medium.
(B) Co-immunoprecipitation of FMNL3 with Rho GTPases. HEK 293 cells were transfected with myc-tagged full-length FMNL3 and GFP-tagged, constitutively-active Rho GTPases. Rho GTPases were immunoprecipitated using GFP-Trap beads and analyzed by western blotting. FMNL3 interacted with Cdc42 and RhoJ, but not RhoA or Rac1.
(C) ECs were transfected with GFP-FMNL3, trypsinized and allowed to spread on coverslips for 25 min. At this time point, cells formed an AMIS on the dorsal surface, which extended as an F-actin-rich (phalloidin; red) bud above the cell. FMNL3 (green) was highly concentrated to the AMIS.
(D) AMIS presence was quantified in fixed HUVEC at 25 min (n = 6; 100 cells/ experiment/ condition).
(E) Live-imaging of AMIS formation showed that FMNL3 depletion did not affect the initiation of AMIS formation, but significantly delayed disassembly (n=3; 100 cells/ experiment/ condition; see Movie S1)
(F) FMNL3 promotes actin cable formation at the AMIS. The arrangement of F-actin in spreading ECs was visualized using phalloidin staining. In most cells, actin cables radiated from the AMIS. Depletion of FMNL3 significantly reduced the number of cells with cables.
(G) This was quantified further by counting the number of cables radiating from the AMIS. Depletion of FMNL3 significantly reduced cable number; whereas overexpression of FMNL3 shifted the profile towards more cables. Data are means ± SEM (n ≥ 5; ≥ 5 cells/ experiment/ condition).
(H) Overexpression of FMNL3 increases AMIS size. F-actin staining (phalloidin) was used to label the AMIS. Depletion of FMNL3 had no effect on AMIS size; however, overexpression of FMNL3 dramatically increased it. Data are means ± SEM (n ≥ 4; ≥ 5 cells/ experiment/ condition). Bars = 10 µm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****P<0.0001.

Figure 2. FMNL3 regulates podocalyxin targeting to the AMIS.
(A) Confocal imaging of fixed ECs was used to measure recruitment of apical proteins to the AMIS, in FMNL3 depleted cells, relative to control. Data are means ± SEM (n ≥ 3; ≥ 5 cells/ experiment/ condition).
(B) Depletion of FMNL3 had no effect on total podocalyxin expression in ECs.
(C) The recruitment of podocalyxin to the AMIS was quantified in HUVEC transfected with two independent siRNAs targeting FMNL3. Depletion of FMNL3 dramatically reduced podocalyxin recruitment to the AMIS. Podocalyxin was 6-fold enriched at the AMIS during apical polarization and this was significantly reduced on FMNL3 depletion. Panels show representative images. Data are means ± SEM (n ≥ 4; ≥ 5 cells/ experiment/ condition). Bar = 10 µm.
(D) ECs were fixed and stained for microtubules (tubulin; green) and podocalyxin (red). Some alignment of podocalyxin vesicles with microtubules was seen in the periphery of the cell (arrowheads; inset panel). Bar = 10 µm.
(E) ECs were fixed and stained for F-actin (green) and podocalyxin (red). The panels show the AMIS (dashed line) and radiating actin cables. The right-hand panel was imaged using

STED; the left-hand panel by standard confocal. A subset of podocalyxin vesicles align with the actin cables (arrowheads). Bar = 1 µm.
(F) ECs were transfected with GFP-myosin Vb, fixed and stained for F-actin (red). In spreading cells, myosin Vb localized strongly to the AMIS. Bar = 10 µm.
(G) ECs were treated with either 20 µM nocodazole or 10 µM MyoVin-1 for 1 h and then trafficking of podocalyxin to the AMIS was quantified in spreading cells. Data are means ± SEM (n = 5; 5 cells/ experiment/ condition).

Figure 3. RhoJ localizes to the AMIS and is required for effective podocalyxin recruitment.
(A) ECs were transfected with either GFP-Cdc42 or GFP-RhoJ. In spreading cells, both proteins were in small vesicles which clustered near the AMIS. RhoJ showed distinct concentration at the AMIS itself. Bar = 10 µm.
(B) Depletion of RhoJ or Cdc42 significantly reduced the percentage of cells with cables radiating from the AMIS, and the number of cables that formed. Data are means ± SEM (n ≥ 8; ≥ 5 cells/ experiment/ condition).
(C) Depletion of RhoJ significantly inhibited podocalyxin recruitment to the AMIS. Data are means ± SEM (n ≥ 8; ≥ 5 cells/ experiment/ condition).

Figure 4. FMNL3 is required for polarized recruitment of podocalyxin to the apical surface during lumen formation.
(A) ECs were transfected with siRNA targeting FMNL3 and plated into a 3D co-culture assay of angiogenesis. After 5 days, ECs were stained for PECAM (green), podocalyxin (red). Nuclei from the ECs and surrounding fibroblasts are blue. Dashed lines show the positions of optical sections taken through the tubes. PECAM was concentrated at the luminal surface between adjacent ECs. In control cells, optical sections showed tightly-focused podocalyxin staining at the luminal surface, which was lost on depletion of FMNL3. Bar = 10 µm.
(B) Quantification of podocalyxin (red) and PECAM (green) distribution in cross-sections of endothelial tubes. The luminal interface is centered at 6 µm. Control endothelial tubes have a concentration of podocalyxin at the early luminal surface, which is lost on depletion of FMNL3. Data are the means (solid lines) ± SEM (shaded area) from three independent experiments.
(C) The working model.